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State-Selective Metabolic Labeling of Cellular Proteins

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Abstract

Transcriptional activity from a specified promoter can provide a useful marker for the physiological state of a cell. Here we introduce a method for selective tagging of proteins made in cells in which specified promoters are active. Tagged proteins can be modified with affinity reagents for enrichment or with fluorescent dyes for visualization. The method allows state-selective analysis of the proteome, whereby proteins synthesized in pre-determined physiological states can be identified. The approach is demonstrated by proteome-wide labeling of bacterial proteins upon activation of the P_{BAD} promoter and the SoxRS regulon, and provides a basis for analysis of more complex systems including spatially heterogeneous microbial cultures and biofilms.

Cell function and survival rely on the proper regulation of gene expression to ensure that proteins are synthesized in response to internal and external demands. Proteins that execute basic cellular functions (“housekeeping proteins”) are constitutively expressed, while expression of other genes may be restricted to specific physiological states. For instance, in bacterial pathogens, ribosomal proteins may be synthesized continually whereas expression of virulence factors may be limited to a particular stage of infection of a host. These differences are controlled in large part by transcriptional regulation and *cis*-regulatory elements, such as up-stream promoters. The level of transcription from a promoter can reflect cellular demand for the associated protein, and may signify a cell’s physiological state. For example, transcription from the *soxS* promoter in *Escherichia coli* drives expression of the SoxS protein, a transcription factor responsible for directing expression of dozens of genes involved in protection against damage by free radicals; correspondingly, activation of the *soxS* promoter is used as an indicator of cell oxidative stress (1). In other cases, transcriptional activity of a particular promoter may be indicative of cell physiology while having no direct role in the regulation of other genes. For example, expression of SspH1 (a secreted virulence factor) by *Salmonella typhimurium* is limited to bacteria residing within mammalian cells (2); while SspH1 has no known role in the regulation of other *Salmonella* genes, its expression is concurrent with that of other proteins that promote intracellular survival.

Phenotypic heterogeneity is characteristic of complex cellular systems ranging from microbial biofilms to multicellular organisms. Conventional proteomic analysis of such

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systems is of limited value, because it provides only an average protein composition that obscures differences among cells that are in various physiological states. Here we describe a method for “state-selective” analysis of the proteome, in which we selectively tag only those proteins that are made in cells in which specific promoters are active.

We have described previously the bio-orthogonal non-canonical amino acid tagging (BONCAT) strategy for selective enrichment and identification of newly synthesized cellular proteins (3). In procedures similar to those used in isotopic labeling, non-canonical amino acids (ncAAs) bearing azide or alkyne side chains are introduced to cells in a “pulse” during which proteins undergoing active translation are tagged. Tagged proteins are distinguished from those made prior to the pulse through bio-orthogonal ligation of the ncAA side chain to probes that permit their detection, isolation (4), and visualization (5, 6). In 2009, we reported a genetically targeted strategy for confining protein labeling to specific cells within heterogeneous mixtures (7), by using the methionine (Met, Fig. 1a) surrogate azidonorleucine (Anl, Fig. 1a) as the metabolic label. In this approach, we relied on expression of the L13N/Y260L/H301L mutant form of the *E. coli* methionyl-tRNA synthetase (designated NLL-MetRS) to enable cells to use Anl in competition with Met during translation (8). Cells that do not express the mutant synthetase are inert to Anl. In cellular mixtures, only those proteins made in cells that express the mutant synthetase are labeled. Through this approach, proteins synthesized in targeted cells can be selectively isolated from complex mixtures for identification by mass spectrometry or conjugated to fluorescent dyes for *in situ* visualization. Hang and coworkers have used NLL-MetRS to probe the *Salmonella* proteome in the course of infection of mammalian macrophages (9).

Here we describe methods for “state-selective” labeling of cellular proteins. We placed the gene encoding NLL-MetRS under control of two promoters of interest and compared the patterns of protein synthesis observed in active and inactive transcriptional states. We anticipated that when the promoter is inactive (or repressed), NLL-MetRS would not be expressed and cellular proteins would not be subject to Anl labeling (Fig. 1b). However, under inducing conditions where transcription from the promoter is active, NLL-MetRS is expressed and newly synthesized proteins can be tagged with Anl (Fig. 1c).

To demonstrate this approach, we first used arabinose induction of the P_{BAD} promoter to drive expression of the mutant synthetase. The combined negative and positive control of transcription from the P_{BAD} promoter yields an “off/on” genetic switch that permits tightly controlled gene expression in *E. coli* (10). We inserted a DNA sequence encoding NLL-MetRS into a plasmid downstream of the P_{BAD} and *ara* operon regulatory elements. We transformed the resulting construct into *E. coli* cells and compared protein labeling under non-inducing and inducing conditions (Fig. 2a). Following a 10-minute pulse with Anl, cell lysates were subjected to Cu (I)-catalyzed ligation (11) to an alkyne probe (Alkyne-TAMRA, Fig. 1a) for selective modification of Anl-tagged proteins. After separation of proteins by SDS-PAGE, in-gel fluorescence scanning revealed proteome-wide incorporation of Anl in arabinose-induced cells. Proteins from cells grown under non-inducing conditions did not exhibit significant labeling. NLL-MetRS expression, as determined by western blot analysis, was consistent with both P_{BAD} transcriptional behavior and fluorescence detection of Anl-tagged proteins (Fig. 2b).

Next we examined a system in which protein tagging was dependent on activation of the oxidative stress response in *E. coli*. We placed NLL-MetRS expression under control of SoxR, an oxidative stress sensor and transcription factor that is constitutively expressed in inactive form (1, 12). SoxR contains two iron-sulfur clusters that are subject to one-electron oxidation or nitrosylation by superoxide and nitric oxide, respectively (12, 13, 14). SoxR binds the promoter that controls expression of the *soxS* gene; transcription from this

promoter is low when SoxR is in its reduced, inactive state. Upon activation of SoxR by superoxide or nitric oxide, strong expression of SoxS is driven from the *soxS* promoter. The SoxS protein, also a transcription factor, is responsible for activating the cellular response to superoxide and nitric oxide, and coordinates expression of more than forty genes involved in detoxification and oxidative-damage repair.

To synchronize labeling with the oxidative stress response, we placed the NLL-MetRS gene under control of the SoxRS regulon by positioning it directly downstream of the *soxS* promoter (see Supporting Information). The resulting construct was introduced to *E. coli* cells via a low-copy number plasmid such that, in addition to control of chromosomally encoded SoxS, the SoxRS regulon was also responsible for directing expression of NLL-MetRS (Fig. 3a). As described earlier for the P_{BAD} system, we examined promoter-directed protein tagging under both non-inducing and inducing conditions. Because the *E. coli* response to oxidative stress is rapid (with superoxide induced transcripts reaching maximal values within minutes (15)), we examined protein tagging during a short interval immediately following exposure to superoxide. We activated the SoxRS regulon in *E. coli* cells grown in liquid culture by adding the superoxide-generating agent paraquat (PQ) (13). After a 15-minute pulse with Anl, tagged proteins were modified in lysates by strain-promoted conjugation to a dibenzazacyclooctyne (16) (DIBAC)-functionalized fluorescent dye (DIBAC-TAMRA, Fig. 1a) and separated by SDS-PAGE. In-gel fluorescence scanning yielded results analogous to those observed with the P_{BAD} system; activation of the *soxS* promoter with PQ (Fig. 2b) induced proteome-wide tagging of proteins synthesized by *E. coli* in the oxidatively stressed state. Labeling was also detected in proteins from cells treated with nitric oxide (Supplementary Fig. 1). Proteins from cells cultured under non-inducing conditions exhibited minimal evidence of tagging. NLL-MetRS expression, as revealed by western blot analysis (Fig. 3c), was induced by addition of PQ, and the extent of protein labeling was dependent on the PQ dose (Fig. 3d, Supplementary Fig. 3).

We achieved useful levels of protein-tagging specificity in these experiments in spite of low levels of “leaky” expression in both the P_{BAD} and SoxRS systems. Reduction of background labeling required optimization of the relative amounts of Anl and Met in the culture medium; we screened conditions by adjusting the concentration of Anl until negligible tagging was observed in non-induced cultures. Background signal was also reduced by using glucose (for P_{BAD} catabolite repression) or thiamine (an antioxidant that reduces basal levels of SoxR oxidation). Use of low copy-number plasmids was essential for minimizing background labeling; we anticipate that a single copy of the NLL-MetRS gene by chromosomal recombination would also yield satisfactory results (17).

We estimate that, under the conditions used in this study, the extent of replacement of Met by Anl is less than 10%. This estimate was made by comparing the intensities of TAMRA fluorescence from cells labeled with the SoxRS system to those from cells labeled under conditions previously shown to yield 10% substitution (Supplementary Fig. 2). Similar results were obtained for the arabinose system (data not shown).

The work described here shows that controlled expression of NLL-MetRS can be used to restrict protein labeling to cells that are in pre-determined physiological states. The linking of protein tagging to promoter activity permits “state-selective” analysis of the proteome such that proteins synthesized in specific cell states can be tagged and identified. Protein tagging is rapid, permitting analysis with temporal resolution in the range of a few minutes. We are currently exploring the use of such methods to interrogate spatially heterogeneous biofilms and multicellular organisms.

METHODS

Materials

AnI was synthesized as previously described (18), but using Na-boc-lysine as the starting material instead of Na-boc-diaminobutyric acid. The [tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) ligand was synthesized as previously described (11). Structures were verified by mass spectrometry and ^1H -NMR spectroscopy.

TAMRA-alkyne and DIBAC-TAMRA were purchased from Click Chemistry Tools. Anti-PentaHis monoclonal antibody AlexaFluor647-conjugate was purchased from Qiagen. Coomassie Colloidal Blue staining kit was purchased from Invitrogen. BCA Protein Assay Kit was used to measure protein concentrations and was from Pierce. pREP4 encoding the bacterial *lacIq* gene was obtained from Qiagen (not to be confused with the commercially available mammalian pREP4). Biotin-PEG-alkyne was purchased from Invitrogen. pACYC was obtained from New England Biolabs free of cost. All other reagents were purchased from Sigma and used without further purification.

Cloning

All cloned constructs were confirmed by DNA sequencing by Laragen before use. Cloning of pJTN1 was previously described (7). To clone pBAD33-NLL-MetRS, the gene encoding an N-terminally His-tagged NLL-MetRS was amplified by PCR from pQE80-NLL (8) using the following primers: 5'-atatggtaccctatcatttagagcttcacc-3' and 5'-atatgagctcgccacgaaggccaggagtgaacgatgagagatcgcatc-3'. The resulting fragment was inserted between the *SacI* and *KpnI* sites of pBAD18 using standard cloning procedures.

To clone pREP4-*soxS*-NLL, the *soxS* promoter and ribosomal binding site sequence (as encoded on the DH10B *E. coli* genome) was added to the 5' end of the N-terminally His-tagged NLL-MetRS by PCR using primer overhangs. The fragment was inserted into the *NheI* site of pREP4. While this construct exhibited leaky expression, NLL-MetRS could still be activated using paraquat and nitric oxide (this construct was used in preparing Supplementary Fig. 1). Because of leaky expression, this construct was not used in subsequent experiments.

To clone pSOX-NLL-MetRS, the IDT_Sox_Regulon sequence (below) was custom synthesized by Integrated DNA Technologies and received from the manufacturer on pSOX_IDT. The gene encoding NLL-MetRS was amplified from pQE80-NLL by PCR using primers: 5'-atagtgatccatgactcaagtcgcaaga-3' and 5'-ggtggaagcctctaaatgaagatct-3'. The resulting fragment was inserted between the *BamHI* and *BglII* sites of pSOX_IDT to form pSOX_IDT_NLL. The cassette encoding the NLL-MetRS under control of the SoxRS regulon (see Supporting Information) was amplified from pSOX_IDT_NLL by PCR using the primers: 5'-atatgctagccccgtgtaaacgacggccagt-3' and 5'-atatgctagcagtattgacctcaggaaacagctatgac-3'. The resulting fragment was inserted at the *NheI* site of the low-copy number pACYC177 plasmid to form pSOX-NLL-MetRS. DH10B *E. coli* cells harboring the pSOX-NLL-MetRS plasmid were used to generate the data displayed in Fig. 2, Supplementary Fig. 2, and Supplementary Fig. 3. A schematic of the construct is shown in Fig. 2a.

Protein Tagging with AnI

P_{BAD} system: DH10B *E. coli* cells transformed with pBAD33-NLL-MetRS were grown in LB medium supplemented with 0.2% glucose and 100 mgL⁻¹ of ampicillin overnight at 37 °C with orbital shaking at 250 rpm. The following day, the culture was diluted 1:50 into M9 minimal medium supplemented with 40 mgL⁻¹ of each of the twenty canonical amino acids,

1 mM MgSO₄, 0.1 mM CaCl₂, 30 mgL⁻¹ thiamine hydrochloride, and 0.4% (v/v) glycerol. This culture was grown at 37°C with orbital shaking at 250 rpm until OD₆₀₀ was ~ 0.5, at which point the culture was divided into two. To one culture, arabinose was added to a final concentration of 0.2% for induction of the NLL-MetRS. Ten minutes later, both cultures were pulsed by addition of AnI to a final concentration of 100 μM. After a 10 m pulse, cells were pelleted by centrifugation at 4 °C, rinsed once with cold PBS, and pelleted again. Cell pellets were frozen at – 20 °C until further processing.

SoxRS system—DH10B *E. coli* cells transformed with pSOX-NLL were grown overnight in SOB medium supplemented with 30 mgL⁻¹ thiamine hydrochloride and 100 mgL⁻¹ ampicillin at 37 °C with orbital shaking at 250 rpm. The following day, the culture was diluted 1:50 into fresh SOB medium supplemented with 10 mgL⁻¹ thiamine hydrochloride and 100 mgL⁻¹ ampicillin and grown at 37°C with orbital shaking at 250 rpm. When the culture reached an OD₆₀₀ of ~ 1.0, the culture was diluted 1:10 into RPMI medium supplemented with 10% (v/v) fetal bovine serum and 10 mgL⁻¹ thiamine hydrochloride (RPMI was selected since this it is a common medium for examining macrophage-induced oxidative stress in *E. coli* (14)). This culture was allowed to grow for 1 h. The culture was divided into two and paraquat was added to one culture at a final concentration of 100 μM (from a freshly prepared 10 mM stock in water). Ten minutes later, both cultures were labeled by addition of AnI to a final concentration of 125 M. After a 15 m pulse, cells were pelleted by centrifugation at 4 °C, rinsed once with ice cold PBS and pelleted again. Cell pellets were frozen at – 20 °C until further processing.

For Supplementary Fig. 1, DH10B cells were transformed with pREP4-*soxS*-NLL and maintained in 35 mgL⁻¹ kanamycin. Paraquat treatment was done as described for pSOX-NLL, and exposure of cells to nitric oxide was done as described by Ding *et al* (13).

Protein Conjugation and Detection

For protein conjugation to alkyne-TAMRA, cells were lysed by suspension in PBS containing 1% (w/v) SDS and heated to 75 °C. Lysates were briefly sonicated to shear DNA and reduce the viscosity of the solution. Protein concentrations were normalized by BCA assay. The conjugation reaction was performed according to the protocol suggested by Hong *et al* (10) using 10 μM alkyne-TAMRA and a 20 m reaction time. Reactions were stopped by addition of AnI to a concentration of 100 μM followed by brief vortexing.

For protein conjugation to DIBAC-TAMRA, cells were lysed by suspension in 100 mM Tris buffer (pH 8.0) containing 1% (w/v) SDS, and heated to 75 °C. Lysates were briefly sonicated to shear DNA and reduce the viscosity of the solution. Prior to addition of DIBAC-TAMRA, cysteine thiols were blocked by addition of iodoacetamide to a final concentration of 25 mM. The blocking reaction was allowed to proceed for 1 h in the dark with mild agitation. Protein conjugation reactions were initiated by addition of DIBAC-TAMRA to a final concentration of 20 μM (from a 5 mM stock in DMSO). Reactions were allowed to proceed for 20 m with mild agitation at room temperature, protected from light. Reactions were stopped by addition of AnI to 100 μM followed by brief vortexing. For detection, ~ 10 μg of protein was separated on 12% Tris-Tricine SDS-PAGE gels. Following electrophoresis, gels were imaged by fluorescence scanning on a GE Typhoon Trio+ fluorescence scanner with detection for TAMRA. Subsequently, proteins were stained using the Coomassie Colloidal Blue staining kit according to the manufacturer's protocol. Images of Coomassie-stained gels were also obtained on the GE Typhoon Trio+ using red-laser excitation of the gel and omitting the emission filter.

Imaging

Cultures prepared as described earlier were pulse labeled with 100 μM Anl for 20 m. To halt protein synthesis, cell pellets were washed with cold PBS containing 30 $\mu\text{g mL}^{-1}$ chloramphenicol. Cells were fixed in 3.7% (w/v) formaldehyde at room temperature for 30 m, permeabilized in 0.1% (v/v) Triton-X 100 in PBS at room temperature for 5 m, sedimented by centrifugation, and resuspended in PBS. Reagents were added to the cell suspension to the following concentrations: 100 μM CuSO_4 , 500 μM THPTA ligand, and 40 μM alkyne-TAMRA. The reaction was initiated by addition of aminoguanidine and sodium ascorbate (from 20X stocks in water) to final concentrations of 5 mM each. The reaction was allowed to proceed for 30 m at room temperature, protected from light. Cells were washed twice with PBS to remove excess dye and resuspended in PBS. Each cell suspension was applied to a 5% agarose slab and covered with a coverslip. Images were obtained with a Zeiss LSM510 confocal microscope using a 63X oil immersion lens. Fluorescence intensities were determined using ImageJ software.

Western Blot Analysis

Protein contents in cell lysates were normalized by BCA assay and 5 μg of protein from each lysate was separated on 12% Tris-Tricine SDS-PAGE gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes and subsequently blocked with 5% milk in PBST. Membranes were probed for NLL-MetRS by addition of Anti-PentaHis AlexaFluor647-conjugate to the blocking buffer at a dilution of 1:5,000. After 1 h incubation with rocking at room temperature, membranes were washed for 5 m with PBST. The wash was repeated three times. Washed membranes were scanned on a GE Typhoon Trio+ fluorescence scanner with detection for AlexaFluor647.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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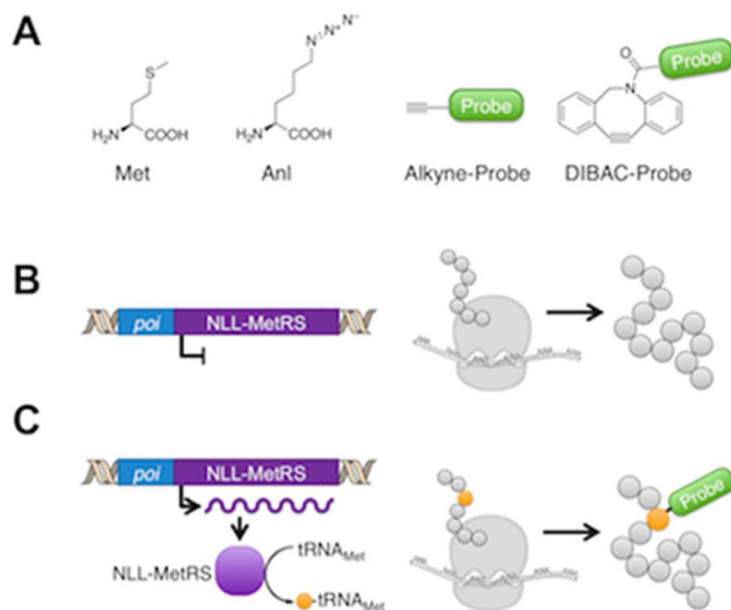


Figure 1. Promoter-directed proteomic labeling with Anl. (a) Structures of amino acids and simplified representations of probes used in this study. (b) NLL-MetRS expression is placed under control of a promoter of interest (poi). When transcriptional activity of the poi is “off,” the NLL-MetRS is not expressed and proteins are not tagged. (c) When transcription of the poi is “on,” the NLL-MetRS is expressed and newly synthesized proteins are tagged.

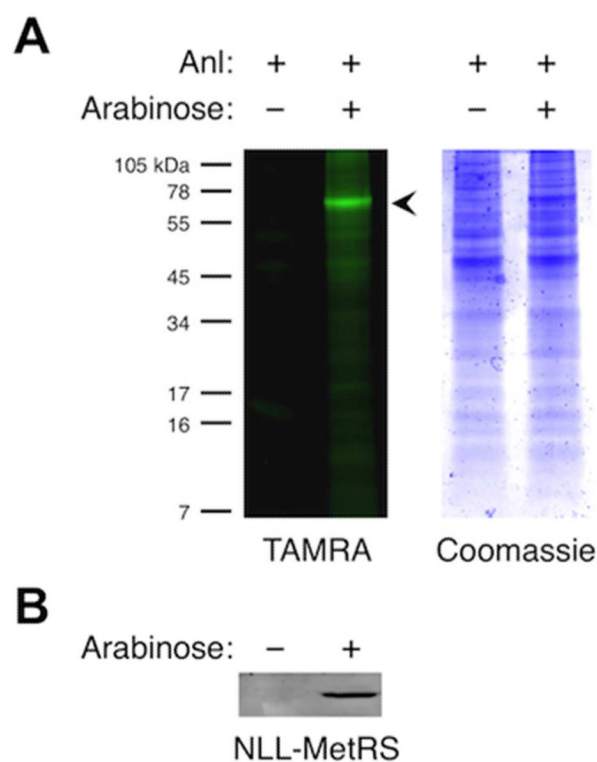


Figure 2.

Proteomic labeling with Anl in response to arabinose. (a) When under control of the PBAD promoter and ara operon regulatory elements, NLL-MetRS can be induced by arabinose. Induced (+ arabinose to 0.2% (w/v)) and uninduced (– arabinose) cells were pulsed with 100 μ M Anl. Incorporation of Anl was assessed by conjugation to alkyne-TAMRA and subsequent detection by in-gel fluorescence scanning (left). Arrowhead indicates labeling of the induced NLL-MetRS. Coomassie staining was used to confirm even loading of protein between lanes (right). (b) Western analysis of NLL-MetRS expression in uninduced (–) and induced (+) cells.

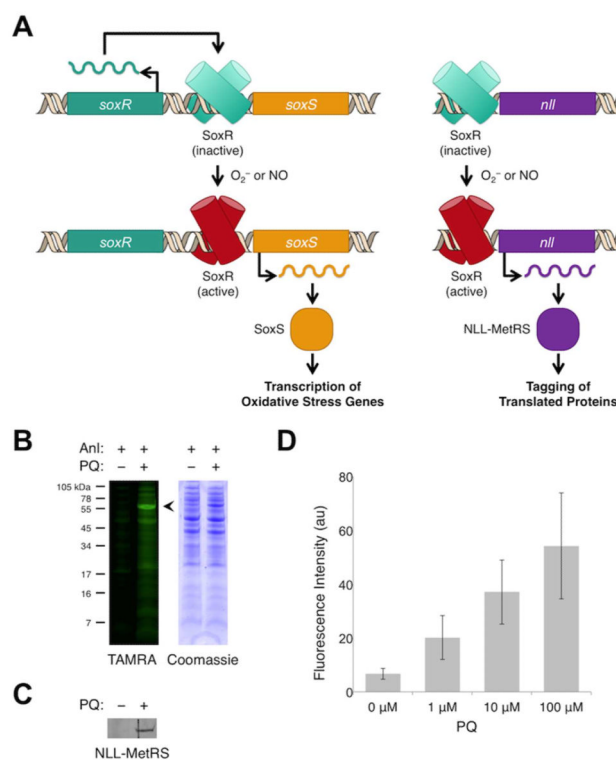


Figure 3.

Proteomic labeling with Anl under conditions of oxidative stress. (a) The NLL-MetRS is under control of the SoxRS regulon and is activated by superoxide or nitric oxide. (b) Anl tagging is activated in cells treated with paraquat (PQ), a superoxide-generating agent. Cells were treated with PQ or were uninduced and subsequently pulsed with 125 μ M Anl. Incorporation of Anl was assessed by conjugation to DIBAC-TAMRA and subsequent detection by in-gel fluorescence scanning (left). Arrowhead indicates labeling of the induced NLL-MetRS. Coomassie staining was used to confirm even loading of protein between lanes (right). (c) Western analysis of NLL-MetRS expression in induced (+PQ) and uninduced (–PQ) cells. (d) The degree of tagging is sensitive to the level of oxidative stress induction by PQ. Cells were treated with various concentrations of PQ and pulsed with a fixed concentration of Anl (125 μ M). The extent of tagging was assessed by conjugation to alkyne-TAMRA and measurement of individual cell intensities by fluorescence microscopy. Error bars represent the standard deviation of each population examined. 140×173mm (150 × 150 DPI) Transcriptional activity from a specified promoter can provide a useful marker for the physiological state of a cell. Here we introduce a method for tagging proteins made in cells in which specified promoters are active. Tagged proteins can be modified with affinity reagents for enrichment or with fluorescent dyes for visualization. The method allows “genetic dissection” of the proteome, whereby proteins synthesized in pre-determined physiological states can be identified.